single type of oligonucleotides over their entire surface or in arrays of multiple types of oligonucleotides spotted with a commercial microarrayer. Nanoparticles having indicator oligonucleotides attached to them and synthetic 30-mer oligonucleotide targets (based on the anthrax protective antigen sequence) were then cohybridized to these substrates (see Figure 32). Therefore, the presence of nanoparticles at the surface indicated the detection of a particular 30-base sequence. At high target concentrations (≥ 1 nM), the high density of hybridized nanoparticles on the surface made the surface appear light pink (see Figure 33). At lower target concentrations, attached nanoparticles could not be visualized with the naked eye (although they could be imaged by field-emission scanning electron microscopy). In order to facilitate the visualization of nanoparticles hybridized to the substrate surface, a signal amplification method in which silver ions are catalytically reduced by hydroquinone to form silver metal on the slide surface was employed. Although this method has been used for enlargement of protein- and antibody-conjugated gold nanoparticles in histochemical microscopy studies (Hacker, in *Colloidal Gold: Principles, Methods, and Applications*, M. A. Hayat, Ed. (Academic Press, San Diego, 1989), vol. 1, chap. 10; Zehbe et al., *Am. J. Pathol.* **150**, 1553 (1997)) its use in quantitative DNA hybridization assays is novel (Tomlinson et al., *Anal. Biochem.*, **171**:217 (1988)). Not only did this method allow very low surface coverages of nanoparticle probes to be visualized by a simple flatbed scanner or the naked eye (Figure 33), it also permitted quantification of target hybridization based on the optical density of the stained area (Figure 34). Significantly, in the absence of the target, or in the presence of noncomplementary target, no staining of the surface was observed, demonstrating that neither nonspecific binding of nanoparticles to the surface, nor nonspecific silver staining, occurs. This result is an extraordinary feature of these nanoparticle-oligonucleotide conjugates which enables ultra-sensitive and -selective detection of nucleic aicds.

It has been determined that the unique hybridization properties of oligonucleotide-functionalized nanoparticles of the present invention can be further used to improve the selectivity of combinatorial oligonucleotide arrays (or "gene chips") (Fodor, *Science* **277**,

393 (1997)). The relative ratio of target hybridized to different elements of an oligonucleotide array will determine the accuracy of the array in determining the target sequence; this ratio is dependent upon the hybridization properties of the duplex formed between different capture strands and the DNA target. Remarkably, these hybridization properties are dramatically improved by the use of nanoparticle labels instead of fluorophore labels. As shown in Figure 35, the dehybridization of nanoparticle-labeled targets from surface-bound capture strands was much more sensitive to temperature than that of fluorophore-labeled targets with identical sequences. While the fluorophore-labeled targets dehybridized from surface capture strands over a very broad temperature range (first derivative FWHM = 16 °C), identical nanoparticle-labeled targets melted much more sharply (first derivative FWHM = 3 °C). It was anticipated that these sharpened dissociation profiles would improve the stringency of chip-based sequence analysis, which is usually effected by a post-hybridization stringency wash. Indeed, the ratio of target hybridized to complementary surface probes to that hybridized to mismatched probes after a stringency wash at a specific temperature (represented by the vertical lines in Figure 35) is much higher with nanoparticle labels than fluorophore labels. This should translate to higher selectivity in chip detection formats. In addition, nanoparticle labels should increase array sensitivity by raising the melting temperature (T_m) of surface duplexes, which lowers the critical concentration below which duplexes spontaneously melt at room temperature.

In order to evaluate the effectiveness of nanoparticles as colorimetric indicators for oligonucleotide arrays, test chips were probed with a synthetic target and labeled with both fluorophore and nanoparticle indicators. The test arrays and oligonucleotide target were fabricated according to published protocols (Guo et al., *Nucl. Acids Res.*, **22**:5456 (1994); arrays of 175 μm diameter spots separated by 375 μm were patterned using a Genetic Microsystems 417 Microarrayer). Arrays contained four elements corresponding to the each of the four possible nucleotides (N) at position 8 of the target (see Figure 32). The synthetic target and either fluorescent-labeled or nanoparticle-labeled probes were hybridized stepwise to arrays in hybridization buffer, and each step was followed with a stringency buffer wash

at 35 °C. First, 20 μL of a 1 nM solution of synthetic target in 2 X PBS (0.3 M NaCl, 10 mM NaH_2PO_4/Na_2HPO_4 buffer, pH 7) was hybridized to the array for 4 hours at room temperature in a hybridization chamber (Grace Bio-Labs Cover Well PC20), and then washed at 35°C with clean 2 X PBS buffer. Next, 20 μL of a 100 pM solution of oligonucleotide-functionalized gold nanoparticles in 2 X PBS was hybridized to the array for 4 hours at room temperature in a fresh hybridization chamber. The array was washed at 35°C with clean 2 X PBS, then twice with 2 X PBN (0.3 M $NaNO_3$, 10 mM NaH_2PO_4/Na_2HPO_4 buffer, pH 7). Then, the nanoparticle arrays were immersed in a silver amplification solution (Sigma Chemical, Silver Enhancer Solution) for 5 min and washed with water. Silver amplification darkened the array elements considerably, and 200 μm diameter elements could be easily imaged with a flatbed scanner or even the naked eye.

Arrays challenged with the model target and nanoparticle-labeled probes and stained with the silver solution clearly exhibited highly selective hybridization to complementary array elements (Figure 36A). Redundant spots of the same capture sequence showed reproducible and consistent hybridization signal. No background adsorption by nanoparticles or silver stain was observed; the image greyscale value reported by the flatbed scanner is the same as that observed for a clear microscope slide. The darker spots corresponding to adenine at position 8 (N=A) indicate that oligonucleotide target hybridized preferentially to perfectly complementary capture strands over mismatched ones, by a greater than 3:1 ratio. In addition, integrated greyscale values for each set of spots follows the predicted stability of the Watson-Crick base pairs, A:T > G:T > C:T > T:T (Allawi et al., *Biochemistry* **36**, 10581, (1988)). Normally, G:T mismatches are particularly difficult to discriminate from A:T complements (Saiki et al., in *Mutation Detection*, Cotton et al., eds. (Oxford University Press, Oxford, 1998), chap. 7; S. Ikuta et al., *Nucl. Acids Res.* **15**, 797 (1987)), and the distinction of these two array elements demonstrates the remarkable resolving power of nanoparticle labels in single nucleotide mismatch detection. The selectivity of the nanoparticle-based arrays was higher than that of the fluorophore-indicated arrays, Figure 36B; fluorophore labels provided only 2:1 selectivity for adenine at position 8.